Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
24 MAR 2009		**00-00-2009 to 00-00-2009**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Antibodies to squalene in US Navy Persian Gulf War veterans with chronic multisymptom illness	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Naval Health Research Center,Department of Defense Center for Deployment Health Research,140 Sylvester Rd,San Diego,CA,92106-3521**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**7**	

Vaccine 27 (2009) 3921–3926

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Antibodies to squalene in US Navy Persian Gulf War veterans with chronic multisymptom illness

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ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form 24 March 2009
Accepted 29 March 2009
Available online 3 May 2009

Keywords:
Immunologic adjuvants
Squalene
Anthrax vaccines

ABSTRACT

Since the end of the 1991 Gulf War, there have been reports of unexplained, multisymptom illnesses afflicting veterans who consistently report more symptoms than do nondeployed veterans. One of the many possible exposures suspected of causing chronic multisymptom illnesses Gulf War veterans is squalene, thought to be present in anthrax vaccine. We examined the relationship between squalene antibodies and chronic symptoms reported by Navy construction workers (Seabees), $n = 579$. 30.2% were deployers, 7.4% were defined as ill, and 43.5% were positive for squalene antibodies. We found no association between squalene antibody status and chronic multisymptom illness ($p = 0.465$). The etiology of Gulf War syndrome remains unknown, but should not include squalene antibody status.

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1. Introduction

After the 1991 Gulf War, many veterans reported health problems that remain unexplained. Although the war was comparatively short-lived, soon after several studies described chronic, nonspecific, multisymptom illnesses in veterans who believed their illnesses were secondary to war-related exposures [1–3]. Hallmarks of the multisymptom illnesses included symptoms such as fatigue, neurocognitive complaints, and musculoskeletal pain.

Multiple vaccine administration and, in particular, vaccination against anthrax, was suggested as a possible etiology [4]. Suspicion of anthrax vaccination increased considerably after the publication of independent research from the private sector that suggested the multisymptom illnesses were consistent with autoimmune disease presentations, likely triggered by exposure to squalene. The published research was careful to state that the "...laboratory-based investigations do not establish that squalene was added as adjuvant to any vaccine used in military or other personnel who served in the Persian Gulf War Era". This study suggested that an immune response to squalene was involved in the pathogenesis, although the no known exposure to a squalene adjuvant was ascertained or reported from the study subjects [5]. The study was subsequently recognized as "inconclusive" by the Institute of Medicine [6]. Although the United States General Accounting Office published a specific and detailed account of anthrax vaccine development and Department of Defense policy regarding the use of adjuvants [7], concerns persisted regarding the potential for vaccine-induced illnesses [8].

In subsequent years, validated assays for squalene antibodies were developed [9–11]. It was proposed that such assays might be applied to stored sera from a population previously surveyed for multisymptom illness after 1991 Gulf War deployment. We therefore proposed a blinded test of the relationship, if any, between squalene antibodies and chronic multisymptoms reported by Navy construction workers.

2. Methods

2.1. Study population

Navy mobile construction battalion personnel (Seabees) build and maintain US Navy and Marine Corps bases, ports, field deployment facilities, and foreign embassies. Before, during, and after the 1991 Gulf War, many Seabees worked throughout Saudi Arabia, preparing airports, building ammunition supply points, constructing roads, and improving the living conditions of other deployed troops [12]. They often worked in small teams and experienced many unique environmental and geographical exposures. Active-duty Seabees who remained in the US Navy after the war and were serving at one of two large Seabee Centers were selected as a study population. This population had several advantages, as previously

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0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2009.03.091

described by Gray et al. [3]. Members of 14 regular active-duty Navy Seabee commands at Port Hueneme, CA, and Gulfport, Mississippi, who had served from September 1990 until the time of the survey in 1994 were eligible for this study. Gulf War service was determined by response to a question regarding military service in the Persian Gulf during Operations Desert Storm or Desert Shield [13].

2.2. Data collection

The study was approved by the Institutional Review Board of the Naval Health Research Center, San Diego, CA, and endorsed by the Institute of Medicine, Washington, DC [14]. It was conducted in compliance with all applicable federal regulations governing the protection of human subjects in research. In late 1994 and early 1995, epidemiologic teams made three visits to each of the two Seabee Centers. Written informed consent was obtained from each participant. The study included an eight-page questionnaire, and the donation of clinical specimens (sera and whole blood) [3]. The questionnaire was introduced by research staff and self-completed by the study subjects. Clinical specimens were preserved at −70 °C. The questionnaire collected information regarding pre-war medical history, war exposures, and symptoms occurring for 1 or more months since July 1990. A second follow-up questionnaire was mailed out between May 1997 and May 1999, which collected responses regarding current symptoms, current health status, health-compromising behaviors, and participation in either of the two federally sponsored Gulf War veteran registries [13]. Exposure and symptom questions were based on the deployment activities of Gulf War veterans and lists of potential exposure risk factors and symptoms [15–17]. Questions were also included to screen for posttraumatic stress disorder and chronic fatigue syndrome.

2.3. Exclusion/inclusion criteria

Potential subjects were excluded if they reported bad reactions to immunizations or injections, or reported cancer, tumors, lung disease, hepatitis, neurological problems, digestive disease, or psychiatric illness. Additional exclusions included self-report of leishmaniasis, HIV infection or AIDS, malaria, any psychological disorder, sleep apnea, narcolepsy, thyroid disorders, or mononucleosis that resulted in at least a 1-week loss from work or school since age 16. Females were also excluded due to the small numbers of participants.

Subjects who reported unusual fatigue and at least three of the additional 38 symptoms listed in Table 1 were defined in these analyses as “ill.” Those without any reported symptoms were categorized as “well”.

Table 1
Self-reportable symptom choices included in the Seabee questionnaire (1997–1999).

Severe headache	Nightmares or flashbacks
Diarrhea	Crying spells
Rash or skin ulcer	Mouth sores
Joint pains	Fever
Joint swelling	Night sweats
Sore throat	Swollen glands/lymph nodes
Generalized muscle weakness	Tender glands/lymph nodes
Confusion	Sudden unexplained hair loss
Forgetfulness	Asthma
Unusual anger	New allergies to medications, foods, odors, or chemicals
Unusual irritability	Inflammation of the eye
Depression	Loss of interest in work, hobbies, sex, or life in general
Chronic worry or anxiety	Chest pain
Appetite loss	Abdominal pain
Suicidal thoughts	Shortness of breath
Unusual muscle pains	Constipation
Cough	Sleepy all the time

Note: Subjects were classified as having chronic multisymptom illness if they self-reported unusual fatigue in conjunction with three or more of the above symptoms.

2.4. Laboratory methods

2.4.1. Materials

Human myelomas (American Type Culture Collection, Manassas, VA) were grown as per the supplier’s instructions with culture medium and additives (Invitrogen). The human antibody secreting myelomas were SA13 (IgG to tetanus toxoid) [18], C5 (IgM to lipid A Gram-negative bacteria) [19], and RPMI 1788 (IgM to tumor necrosis factor beta) [20]. Other agents included pooled human serum (United States Biological, Swampscott, MA), peroxidase-linked sheep anti-human IgG (γ-chain specific) and anti-human IgM (The Binding Site Inc., San Diego, CA), and ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

2.4.2. ELISA assay for antibodies to squalene in human serum

Squalene (SQE; Sigma–Aldrich Chemical Company, St. Louis, MO) was diluted in isopropanol (ISP; T. Baker, Phillipsburg, NJ) and (0.2 μmol/ml; 9.6 μl SQE/100 ml) and 0.1 ml (20 nmol) was placed in each well of a Costar 96-well round bottom tissue culture plate (Corning Inc., Corning, NY). Control wells contained ISP alone. The plates were placed in a biological safety cabinet and incubated overnight to allow the ISP to evaporate. PBS–0.5% boiled casein (T. Baker), pH 7.4 (0.3 ml) was added to each well. After incubation at room temperature for 2 h, the buffer was removed and the plates were tapped on paper towels to remove the residual blocker. Human serum was diluted serially in PBS–0.5% casein starting at a 1:25 dilution. Diluted serum (0.1 ml/well) was added to the plate in triplicate. The plates were covered with plastic wrap and incubated overnight at room temperature. The plates were then washed four times with 0.5 ml/well of PBS (10× Dulbecco’s PBS without Ca^{2+} and Mg^{2+}; Invitrogen Corporation, Carlsbad, CA), pH 7.4. Peroxidase-linked sheep anti-human IgG (γ-chain specific) and IgM (μ-chain specific) was diluted 1:1000 in PBS–0.5% casein, and 0.1 ml/well was added to the plate. Following a 1-h incubation at room temperature, the plates were washed as described above. ABTS substrate (0.1 ml/well) was added and the plates were incubated at room temperature for 1 h. Absorbance was read at 405 nm. Each plate contained culture supernatant from the C5 cell line, pooled normal human serum, and blocking buffer as controls. It should be noted that polystyrene pipettes, tubes, or other objects containing polystyrene, were not used in this assay. The use of polystryrene causes high background (ISP) absorbances and would have greatly increased the variability of the results. Highly purified ISP also was required in order to ensure low background absorbances.

2.4.3. Antibody dilution curve analysis

Sera were defined as positive for antibodies to SQE if two dilutions, 1:25 and 1:50, had absorbances that were greater than three times baseline. Baseline was defined as the absorbance at which the dilution curve became horizontal. We derived the dilution curves by using PROC NLIN within SAS software (SAS Institute, Inc., Cary, NC) to model the absorbance data, the majority of which was symmetric. The four-parameter logistic-log function [21,22] is optical density (OD) $= d + \{(a - d)/[1 + (\text{dilution}/c)^b]\}$ where d is the curve’s lower asymptote or baseline.

2.4.4. Data analyses and statistics

Demographic data, deployment status, squalene antibody status and chronic multisymptom illness status were univariately compared using t-tests for continuous variables and Pearson’s chi-

squared tests for categorical data. Age was determined as of July 31, 1991. Odds ratios and 95% confidence intervals were determined using Cornfield or exact methods. Kappa agreement statistics were calculated for selected questions among individuals who completed the survey twice [3]. All statistical analyses were performed with SAS.

3. Results

We enrolled 970 nondeployed veterans and 527 Gulf War veterans. No differences were found between enrolled participants and nonparticipants with respect to age group, race/ethnicity, marital status, or high school graduation rates.

Sufficient sera were not available for 151 subjects therefore we analyzed 1346 veteran questionnaires and subsequently removed 144 subjects who met the protocol's exclusion criteria. Another 371 subjects were excluded as they had very few symptoms and did not meet the complete case definition for "ill," leaving 831 subjects who met all the inclusion criteria. Of those, 236 produced invalid antibody dilution curves (i.e., no convergence secondary to incomplete or scattered raw data). The remaining 595 had valid dilution curves. Only 16 female subjects remained (2.7%) and were removed, decreasing the number of covariates, producing a final analysis set with 579 subjects (see Fig. 1).

The demographic characteristics of squalene abx-negative veterans and squalene abx-positive veterans were similar when comparing age, race/ethnicity, marital status, and education. Among the 579 cohort subjects, 43 (7.4%) met the criteria for "ill". Comparing demographic characteristics, the mean age, race distribution, marital status and education were not statistically different for well veterans versus ill veterans (Table 2).

For ill subjects (Table 3), deployers outnumbered nondeployers by approximately 2:1 (67.4% vs. 32.6%, respectively (odds ratio [OR], 5.5; 95% confidence interval [CI], 2.8–10.8).

Ill veterans had nearly equal squalene abx status proportions; 51.2% abx-negative vs. 48.8% abx-positive. Statistically, there was no significant association between squalene abx status and CMI status ($p = 0.465$ chi sq.) (Table 4).

We wanted to examine further whether prior deployment had any effect on the lack of association between squalene abx status and chronic multisymptom illness. Stratification by deployment history did not reveal any association (Table 4). Gulf War veterans had statistically similar proportions of squalene abx positive or negative prevalences of chronic multisymptom illness ($p = 0.708$ chi sq.), as did nondeployers ($p = 0.748$ chi sq., or $p = 1.000$ Fisher's Exact Test).

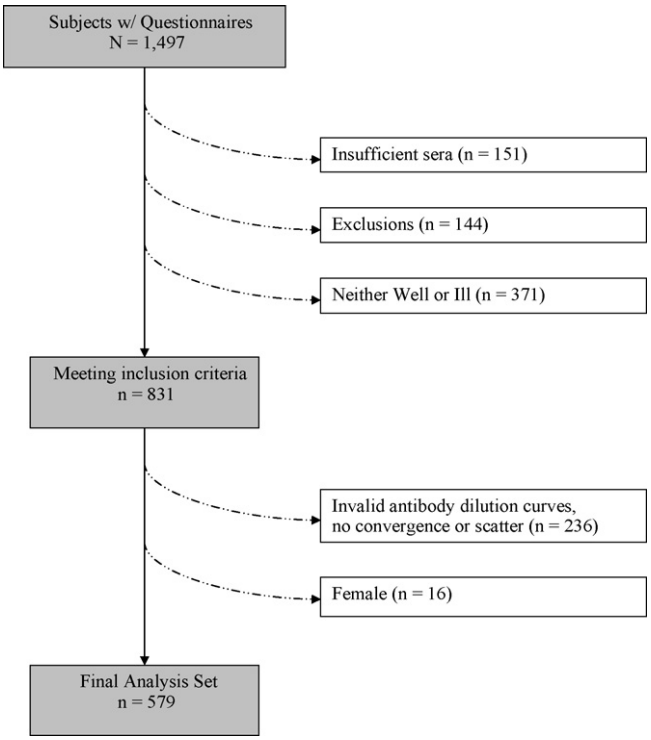

Fig. 1. Schematic diagram illustrating exclusions for analysis of US Navy Seabees with squalene.

4. Discussion

Since the end of the 1991 Gulf War, there have been reports of unexplained, multisymptom illness afflicting veterans who served in that conflict [23].

Table 2
Demographic characteristics by squalene antibody status and CMI status.

	Squalene antibody ($N = 579$)						CMI ($N = 579$)					
	Negative			Positive			Well			Ill		
	n	(%)[a]	x	n	(%)[a]	$\bar{x}$	n	(%)[a]	x	n	(%)[a]	$\bar{x}$
Total	327	(56.5)		252	(43.5)		536	(92.6)		43	(7.4)	
Mean age			28.8			29.1			28.5			25.9
Race												
White non-Hispanic	251	(76.8)		202	(80.2)		417	(77.8)		36	(83.7)	
Black non-Hispanic	28	(8.6)		14	(5.6)		40	(7.5)		2	(4.7)	
Other	44	(13.5)		32	(12.7)		73	(13.6)		3	(7.0)	
Unknown[b]	4	(1.2)		4	(1.6)		6	(1.1)		2	(4.7)	
Marital status												
Married	115	(35.2)		76	(30.2)		172	(32.1)		19	(44.2)	
Single	206	(63.0)		175	(69.4)		357	(66.6)		24	(55.8)	
Unknown[b]	6	(1.8)		1	(0.4)		7	(1.3)		0	(–)	
Education												
High school or less	171	(52.3)		138	(54.8)		286	(53.4)		23	(53.5)	
More than high school	135	(41.3)		101	(40.1)		218	(40.7)		18	(41.9)	
Unknown[b]	21	(6.4)		13	(5.2)		32	(6.0)		2	(4.7)	

Abbreviation: CMI, chronic multisymptom illness. *Note*: Findings are not statistically significant.

[a] Percents may not add to 100 due to rounding.

[b] Unknown status assigned when survey question was left blank.

Table 3
CMI status by deployment status[a].

	Study population ($N = 579$)			
	CMI			
	Well		Ill	
	n	(%)	*n*	(%)
Total	536	(92.6)	43	(7.4)
Deployed				
No	390	(72.8)	14	(32.6)
Yes	146	(27.2)	29	(67.4)

Abbreviation: CMI, chronic multisymptom illness.
[a] Odds ratio, 5.53; 95% confidence interval, 2.84–10.8; $p < 0.0001$.

A range of potential exposures have been postulated or suspected as risk factors for this illness: depleted uranium [6,24], nerve gas [25,26], organophosphates [27,28], vaccines [29], and bacterial infections [30]. In particular, US Navy Seabees have been among the most symptomatic and studied Gulf War veterans [3,13,31–33].

Weak associations have been uncovered, but no single or group of exposures have been identified as strongly associated with chronic multisymptom illness [3,34]. One of the many possible exposures suspected of causing chronic multisymptom illness and other health problems in Gulf War veterans was squalene. One group of researchers hypothesized that squalene in predeployment vaccines triggered an autoimmune disease that could explain many of the symptoms experienced by Gulf War-era veterans [5]. However, this study was flawed because an external exposure to squalene (presumably from vaccination in preparation for deployment) was not definitively ascertained, and therefore, not confirmed. These researchers found antibodies to squalene in 95% of 38 ill Gulf War veterans and 100% of six ill nondeployed veterans who received immunizations in preparation for service in the Gulf. This result was compared with the results of 12 healthy Gulf War veterans, none of whom had antibody reactivity to squalene. In a nonblinded screening study, the same researchers also tested the blood of a larger group of 86 Gulf War veterans, not segregated by clinical status, and found that 69% reacted positively to the squalene antibody test. These results remain questionable due to essential methodological flaws, as detailed by the Institute of Medicine [6] and by Alving and Grabenstein who noted the lack of positive and negative controls for the squalene antibody assay used [35].

Using validated assays, we found no association between squalene antibody status and chronic multisymptom illness. For our subjects, the percent positive for squalene IgG antibodies was 44%, falling in the range of two previously reported percentages, 15–79%, in healthy populations [10,36]. Not unexpectedly, there was a significant association between deployment and the likelihood of chronic multisymptom illness (OR, 5.5; 95% CI, 2.8–10.8). We stratified our primary study question by deployment and again found that there was no statistically significant association between squalene antibody status and chronic multisymptom illness, regardless of past deployment history.

After this current investigation was first proposed and initiated, a method of enhanced sensitivity for determining the presence of squalene in anthrax vaccine using high-performance liquid chromatography was applied to 44 bottles from 38 lots of anthrax vaccine. In 43 bottles from 37 lots produced over a 20-year period (1982–2002), no squalene was detected within a detection limit of 1 ng/0.5 ml dose (2 ppb). One lot was found to contain trace amounts of squalene at 7, 9, and 1 $\mu g\,l^{-1}$, levels considerably below normal human plasma levels (290 $\mu g\,l^{-1}$). The overall results of that investigation provided evidence that past and present anthrax vaccine products are nearly free of squalene [37]. Even if the anthrax vaccine or other vaccines for military use had contained squalene in biologically active amounts, it is unlikely they induced an antibody response. Del Giudice et al. [36] recently demonstrated that an influenza vaccine with the MF59 adjuvant (a squalene-in-water emulsion, used in Europe) neither induced anti-squalene antibodies nor augmented preexisting anti-squalene antibody titers; in fact, anti-squalene antibodies were detected frequently at low titers in

Table 4
CMI status, "well" or "ill", by squalene antibody status[a].

	Study Population N = 579			
	CMI			
	Well		Ill	
	n	(%)	n	(%)
Total	536	(92.6)	43	(7.4)
Squalene Antibodies				
Negative	305	(56.9)	22	(51.2)
Positive	231	(43.1)	21	(48.8)

[a] Findings not statistically significant ($p = 0.465$)

Deployed / Non-Deployed

	Deployed Seabees n=175			
	CMI			
	Well		Ill	
	n	(%)	n	(%)
Total	146	(83.4)	29	(16.6)
Squalene Antibodies				
Negative	71	(48.6)	13	(44.8)
Positive	75	(51.4)	16	(55.2)

[a] Findings not statistically significant ($p = 0.708$)

	Non-Deployed Seabees n=404			
	CMI			
	Well		Ill	
	n	(%)	n	(%)
Total	390	(96.5)	14	(3.5)
Squalene Antibodies				
Negative	234	(60.0)	9	(64.3)
Positive	156	(40.0)	5	(35.7)

[a] Findings not statistically significant ($p = 0.748$)

sera from healthy subjects who had no history of any vaccination containing squalene.

This study had a number of limitations. All morbidity and exposure data were self-reported. Our findings should be viewed in light of perceptual and response biases likely to be present in this setting [38,39]. Our previous work [3] and others' [40] has demonstrated that recall bias is a challenge among Gulf War Seabees. It is likely that some Gulf War Seabees were influenced by news reports and past survey participation [41]. These exposures may have caused veterans to report more symptoms than they otherwise might have reported.

Our study had a number of strengths. The survey data source was extracted from the third-largest controlled survey of 1991 Gulf War veterans with preserved sera obtained at the time of survey administration. This large cohort provided a nondeployed comparison group from which to contrast Gulf War veteran symptom histories. We employed a validated, proven assay for squalene antibody status with appropriate controls.

The desire to find an explanation for the chronic multisymptom illnesses that some 1991 Gulf War veterans suffered has been a quest for the patients themselves and for medical researchers in the civilian and military sectors for over a decade. Our primary finding—there is no association between squalene antibody status and chronic multisymptom illnesses—coupled with direct evidence for the absence of squalene in nearly all of the anthrax preparations tested [37], may dissuade further interest in squalene as a likely cause for the signature illness for the 1991 Gulf War.

Acknowledgments

We are indebted to the Navy Seabee study participants. We also thank Gia Gumbs, Anthony Hawksworth, Robert Reed, Steven Spiegel, and James Whitmer from the Department of Defense Center for Deployment Health Research; Michelle Stoia, from the Naval Health Research Center, San Diego, CA; we appreciate the support of the Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, MD.

Funding: This represents Naval Health Research Center report 08-34, supported by the Department of Defense, under work unit No. 60002. Funding sources did not play any other role. The views expressed in this article are those of the authors and do not reflect the official policy or position of the US Department of the Navy, US Department of the Army, US Department of the Air Force, or US Department of Defense, approved for public release. This research has been conducted in compliance with all applicable federal regulations governing the protection of human subjects in research (Protocol NHRC.2002.0013).

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